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Nitric Oxide Generating Polymeric Coatings for Subcutaneous Glucose Sensors

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14. ABSTRACT

Efforts to date to develop implantable sensors for real-time clinical monitoring of glucose subcutaneously (SQ) in diabetic patients have been hindered by the erratic results owing to biocompatibility problems induced by sensor implantation (e.g., inflammatory/foreign body response). The goal of this research program is to explore and optimize the chemistries required to fabricate implantable amperometric glucose sensors with outer polymeric coatings that slowly generate low levels of nitric oxide (NO). Release of NO has been shown to enhance the biocompatibility of the implanted sensors by decreasing the inflammatory response. The focus of this research is to develop new polymeric coatings (biomedical hydrogels and polyurethanes) that possess immobilized copper ion sites or organoselenium and organotellurium species that will serve as catalytic sites for *in situ* conversion of endogenous nitrosothiol species (RSNO) to NO, thereby providing a sustained local generation of NO at the surface of implanted sensors. Preliminary biocompatibility experiments suggest that RSNO levels within the SQ fluid of rats may be sufficient to generate enough local NO to reduce the inflammatory response at the implant site. New needle-type sensors are being developed to determine the levels of RSNOs in the SQ region. Finally, functional needle-type SQ glucose sensors have been prepared with both NO release and NO generation coatings. These sensors provide the basis of assessing if NO generation/releasing chemistries are compatible with electrochemical glucose sensing chemistries.

15. SUBJECT TERMS
Biocompatibility; sensors; inflammation; angiogenesis; polymeric coatings; nitric oxide

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INTRODUCTION

At present, efforts to develop implantable sensors for real-time clinical monitoring of glucose subcutaneously (SQ) in diabetic patients have been limited by the unreliable analytical results that occur largely due to biocompatibility problems induced by sensor implantation (e.g., inflammatory/foreign body response). This Army sponsored research program is aimed at exploring and optimizing the chemistries required to fabricate implantable amperometric glucose sensors with outer polymeric films that slowly generate low levels of nitric oxide (NO) from endogenous S-nitrosothiol (RSNO) species that are likely present in the interstitial fluid. The generation of NO in the SQ region is expected to greatly enhance the biocompatibility of the *in vivo* sensors by decreasing the inflammatory response and promoting angiogenesis as well as wound healing at the implant site.

While previous studies with thin NO release polymer coatings on SQ glucose sensors have already demonstrated that local NO release can reduce initial inflammatory response of the surrounding SQ tissue [1], the reservoir of NO precursors that can be retained in such thin polymeric coatings is low and this has made it difficult to achieve prolonged NO release (>1 d) at physiologically relevant levels. A very recent advance in the design of NO-release sensors during the past year of this project has partially obviated this limitation (see below). Nonetheless, for long-term sensor implants (weeks to months), it is most desirable to develop a completely new strategy to generate NO locally at the surface of the devices once implanted. Hence, the main focus of this research program is to develop new polymeric coatings (biomedical hydrogels and polyurethanes) that possess immobilized copper (II) ion or other sites (e.g., organoselenium or organotellurium) that will serve as catalytic surfaces for *in situ* conversion of any endogenous *S*-nitrosothiol species (e.g., nitrosoglutathione, nitrosocysteine, etc.) to NO, thereby providing a sustained local generation of NO species at the surface of the implanted sensors. Experiments are being undertaken to assess whether these new NO generating polymers can decrease inflammatory response when implanted in the SQ of rats. Finally, functional needle-type glucose sensors are being prepared with the new polymers to determine whether the use of such polymers as outer sensor coatings enable acceptable electrochemical sensitivity and response times for glucose measurements. At the same time, as mentioned above, the original NO release polymer strategy is being revisited via a new sensing configuration that has the potential to enable much longer-term NO release, without compromising the glucose response characteristics of the sensors.

BODY

Progress has been made on several fronts over the past 12-months with respect to the major goals delineated in the original grant application. Our three main objectives are: 1) to prepare and characterize new polymers (e.g., derivatized polyurethanes and various hydrogels)) possessing immobilized copper(II) ion sites (via Cu(II)-cyclen complexes) and potentially other catalysts such as organoselenium (RSe) and organtellurium (RTe) species that can generate NO from RSNO species as more biocompatible sensor coatings; 2) *in vivo* testing of the anti-inflammatory behavior of these coatings within the subcutaneous tissue of rats using non-sensor implants, and 3) fabricate functional needle-type glucose sensors in our laboratory with the new NO generation) coatings, and demonstrate that NO generation chemistry does not decrease the analytical functionality of the electrochemical glucose sensing devices. As the project as progressed, we also became interested in preparing needle-type sensors that will be able to detect the levels of RSNO species in the SQ fluid, since it is unknown (based on current literature) whether SQ RSNO levels are adequate for the NO generating concept to function effectively. In addition, since we have extensive data showing that NO release polymers do function effectively in the SQ region to reduce inflammatory response, it became of interest to assess whether altering the fundamental design of the needle type electrochemical glucose sensor would allow us to implement such coatings to achieve much longer-term NO release than heretofore possible. Accomplishments during the past year of research in these various areas are summarized below.

1)) Synthesis and Characterization of New Polymers/Coatings that Catalytically Generate NO:

Last year we reported the first polyurethanes with covalently attached with Cu(II)-cyclen complex as the coating to generate NO. This work was recently published [2], and we began work on slight methodology changes in the synthetic scheme to make preparation of this type of material more convenient. In addition, during the past year, we successfully prepared a new polyurethane material with covalently linked RSe species, and demonstrated that this new polymer can also generate NO from various RSNOs. The synthetic route used for this new PU material is outlined in Figure 1.

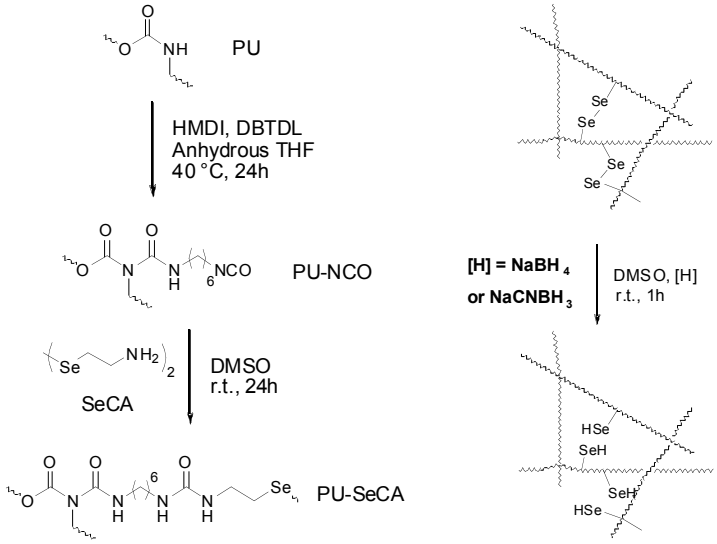

Figure 1. The synthesis scheme used to prepare polyurethanes possessing covalently appended RSe catalytic sites.

Two biomedical grade polyurethanes (Tecophilic and Tecoflex) were chosen as the model polymers for RSe immobilization. They both are thermoplastic poly(ether) polyurethanes but differ in composition of soft segments, hydrophobicity, water uptake, mechanical strength and other properties. The urethane groups on the Tecophilic and Tecoflex backbones were used to couple the polymers with hexamethylene diisocyanate (HMDI) through an allophanate reaction in the presence of a dibutyltin dilaurate (DBTDL) catalyst. The resulting polymer, with pendant free isocyanate groups, was then reacted with amine groups of selenium cystamine (SeCA), a small diselenide compound which was synthesized in our laboratory. The resulting PU-SeCA is then reduced with $NaBH_4$, $NaCNBH_3$ or other reducing agents, such as glutathione, to remove any uncoupled halves of the SeCA molecules.

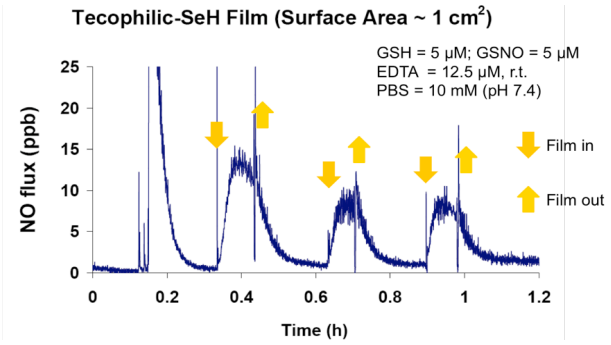

Figure 2. NO generation from piece of RSe-PU material when placed into solution of nitrosoglutathione/glutathione (GSNO/GSH) as measured by chemiluminescence detection of NO.

As shown in Figure 2, when a small disk of the new PU-SeCA polymer is placed into a solution of 5 µM GSNO/GSH in phosphate buffered saline (PBS), pH 7.4, a burst of NO is detected (see Figure 2) and then the NO flux slows to reach a steady-state NO level. When the film is removed from the solution, the NO signal returns to the original baseline, implying that the presence of this polymer initiates the NO liberation from GSNO (*S*-nitrosoglutathione). The repeated insertion/removal of the film demonstrates that the polymer can generate steady-state NO fluxes after each immersion and removal from the test solution (see Figure 2). Decreases in the amount of NO for each immersion is due to consumption of the GSNO substrate. The synthesized Se-PU has good NO generation capability in low concentrations of GSH and GSNO (5 µM). Therefore, this new polymer represents a promising material for coating implantable glucose sensors.

Last year we also reported on the initial feasibility studies of utilizing new RSe catalysts for preparing coatings via a novel layer-by-layer (LbL) deposition method, to make it convenient to coat the catalysts on any sensor surface material. We have utilized polyethylenimine (PEI) as the polymer and covalently linked RSe sites to this polymer. This species is then alternately dip coated with a polyanionic polymer, alginate, to create the LbL coating that is held in place by strong electrostatic interactions between the RSe-PEI and the alginate. During the past year, great improvements the method have been achieved. The coatings have been found to be quite stable, and can generate more NO from RSNOs if a greater number of layers are coated on a given substrate (up

to 100 layers thus far have been tested). A detailed manuscript describing this new technique has now been published [3]. The LbL method will likely become quite attractive for coating glucose sensors with an NO generating surface, and efforts during the coming year will focus on assessing whether this new LbL coating method can provide polymers with improved biocompatibility in the SQ space, and also whether glucose sensors coated with these materials exhibit acceptable analytical performance.

Research also continued regarding the potential for using polymers with immobilized RTe sites to generate NO from RSNOs. A novel ditelluride species was synthesized and covalently linked to poly(allylamine) that was then crosslinked to form a hydrogel type of material [4]. Using chemiluminescence measurements we demonstrated that films of such crosslinked polymers can generate NO from physiological RSNO species, and that they can also be utilized to prepare analytical useful electrochemical RSNO sensors [5]. However, owing to the potential greater toxicity of RTe species (than RSe sites), we do not believe that these types of coatings are preferred to the immobilized Cu(II) or RSe site materials for developing the SQ glucose sensors.

2) In Vivo Testing of NO Generation from Subcutaneous Tissue in Rats: We have reported in the two previous progress reports that there was initially very promising *in vivo* data on the potential for using our new NO generating polymers to reduce inflammatory response in the SQ tissue of rats. However, recent efforts to reproduce those results were stymied by contradictory results, which we concluded might be a mislabeling issue (see last year's report). Animal experiments during the past year to reassess the situation were slowed due to the departure of one key post-doc (Dr. Megan Frost) and our veterinarian collaborator (Dr. Raimon Duran) in August 2007. This necessitated training a new post-doc (Dr. Jason Bennett) to prepare the implants, and a new veterinarian (Dr. Kelly Huginin) to carry out the surgical procedures for implantation and explanting the test polymers under the back skin of rats. In addition, we wanted to modify the procedures slightly from a prior approved animal protocol (with respect to number of implants in each rat (increase)), and this required filing an amendment to our approved UCUCA protocol. Hence, only recently was this amended protocol approved and we were able to restart the *in vivo* evaluation of the new NO generating polymer coatings. For these most recent experiments, we are now awaiting final results from the pathologist who is quantitating the levels of inflammatory cells on the tissue sections removed from the implant sites. Therefore, to more completely evaluate the biocompatibility of the new Cu(II)- and RSe-based polymers in the SQ tissue of rats, we have recently requested and received a 1-year no-cost extension for this project.

3) Preparation of Needle-Type NO and RSNO Sensor: Last year we reported an initial design for needle-type NO and RSNO sensors that could potentially be used to probe the levels of RSNO in the SQ tissue of rats. The NO sensor exhibited good response to NO, with sensitivity of 30-60 nA/μM and a detection limit of 5 nM. This year, the NO sensor has been further optimized (see Fig. 3) by depositing a Ni(II)-(tetrakis-3-methoxy-4-hydroxyphenyl) porphyrin (NiTMHPP) layer, instead of a platinum black film, onto the platinum wire sensing opening in the hope of achieving a smoother surface that would allow improved coating of the outer gas permeable membrane. The sensor is then coated with thin layers of silicone rubber and Teflon AF, and the resulting device has yielded excellent NO sensitivity, high selectivity over NO_2^- and NH_4Cl, and good response times. The sensitivity towards NO ranged from 0.4-3.1 pA/nM, with an average value of 1.1 ± 0.9 pA/nM (n=8). The response time to 95% of the signal was ~120 s, and the limit of detection was 1.7 ± 0.9 nM, though a limit of 0.5 nM was obtained for a few sensors.

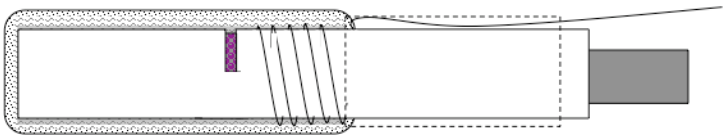

Figure 3. Schematic of *in vivo* NO sensor with "micro" sensing region. Purple in sensing region represents NiTMHPP layer. The layers over the sensor represent PDADM, 1% silicone rubber, and Teflon AF, respectively. This sensor can be coated with polymers containing RSe sites to create a sensor for SQ measurements of RSNO species.

Initial studies to fabricate the needle-type RSNO sensor were also carried out using a SePEI catalyst via our recently reported layer-by-layer deposition method [3]. The NO sensor described above was coated with 40 bi-layers using an automatic dip-coating procedure. Although the signal from such devices was somewhat noisy, the sensor did exhibit a significant response towards GSNO. The sensitivity was 9 pA/μM (r^2 ~0.95), with a response time of 1.5 min and a limit of detection of ~400 nM. The signal was reproducible and reversible and these results are quite promising. It is our hope to further optimize this needle RSNO sensor, and begin to test for the levels of RSNO in the SQ tissue of rats in the coming year.

4) <u>Fabrication of Functional Needle-Type Glucose Sensors</u>: Last year we reported that we began to re-examine the possibility of preparing a longer term (2-3 d) NO release glucose sensor using a relatively simple re-design that allows us to coat much thicker layers of NO release polymers (PurSil, copolymers of polyurethanes (PU) and polydimethylsiloxane (PDMS) doped with lipophilic diazeniumdiolates [6]) (see Fig. 4) without compromising the overall electrochemical response to glucose. The NO release time and the flux level have been optimized during the last 12 months by changing both the amount of diazeniumdialates doped within the PurSil and the layers of PurSil coated onto the sensor. As illustrated in Fig. 4, the thicker coating does not cover a 1 mm window of the sensor directly where the glucose oxidase enzyme is deposited atop the exposed platinum surface coated with Nafion and an electropolymerized copolymer of 1,3-diaminobenzene/resorcinol (to improve sensor selectivity). However, the remaining surfaces of the needle sensor are coated completely with the PU/PDMS material. We have found that this new configuration gives excellent glucose response, while the entire sensor now emits NO for up to 7 days at physiologically relevant fluxes. This design of glucose sensor also has shown excellent response to glucose and performed relatively stable over a one week time period.

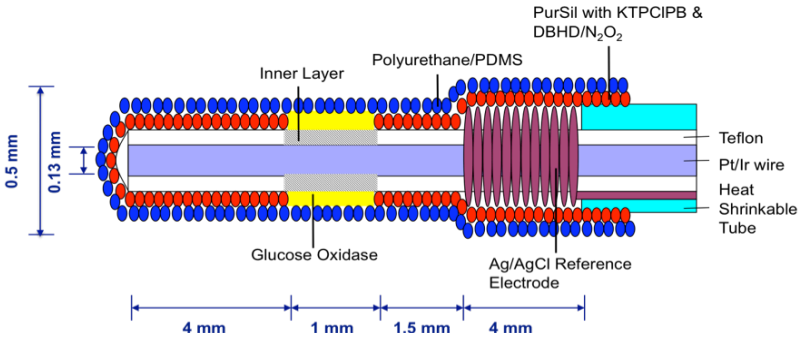

Figure 4. Schematic of new design for needle-type electrochemical glucose sensor with longer-term NO release capability. Thicker coating of NO releasing polymer (PurSil) is over entire surface of sensor, except small window where enzymatic layer resides.

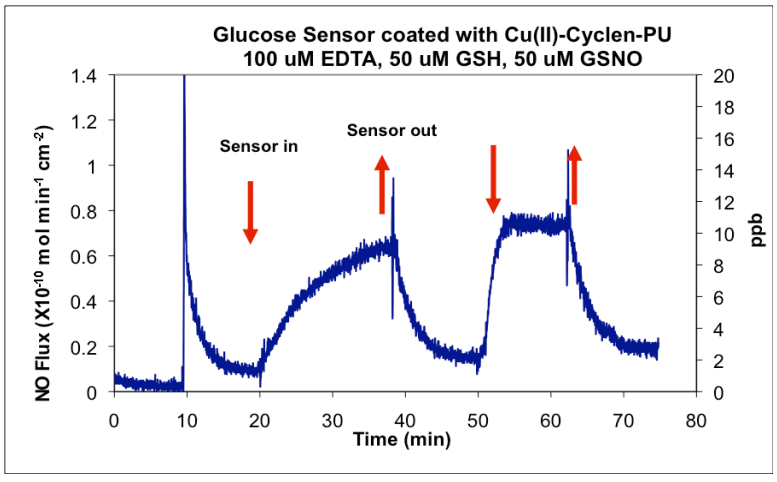

Figure 5. NO generation profile of glucose sensor coated with Cu(II)-cyclen PU.

We also carried out some initial studies of the effect of using different outer polymer coatings that possess the immobilized Cu(II) and RSe sites for generating NO from RSNO species for preparing needle-type glucose sensors. Indeed, now that the PU material has been modified with

immobilized Cu(II)-cyclen complex [2], this material was the first to be tested for preparing NO generating glucose sensors. The whole glucose sensor is covered with a coating of the new polyurethane possessing covalently attached Cu(II)-cyclen species . The resulting sensor is capable of generating physiological level of NO via RSNO species (see Fig. 5). However, the linear range of the glucose sensor is not as wide as that of the NO release glucose sensor because the Cu(II)-cyclen modified polyurethane is more hydrophilic than that used for NO release glucose sensor, make the membrane over the sensing site more permeable to glucose, which creates non-linearity at the high concentration range. Again, using the new configuration shown in Fig. 4 will eliminate this problem, since only plain PU will actually coat the glucose sensing layer, and the NO generating material will be present over the remainder of the device. We plan to prepare NO generating glucose sensors with this new configuration in the coming weeks.

We have successfully developed the layer-by-layer deposition method of SePEI onto various materials including polymers to generate NO from RSNOs. We also still plan to study the effect of using such outer coating onto PU/PDMS coated glucose sensor.

KEY RESEARCH ACCOMPLISHMENTS (during year 3):

- Finalized methodologies to covalently attached Cu(II)-cyclen to various polyurethane matrices and demonstrated that these new materials are able to catalytically generate NO from RSNO species at physiological pH values.

- Synthesized new organoselenium compounds attached to biomedical grade polyurethanes and demonstrated that these materials can generate NO from RSNOs.

- Completed work on novel polymers containing RTe sites, and demonstrated that crosslinked hydrogels of these materials generate NO from RSNOs as effectively as polymers with RSe sites.

- Further optimized fabrication of first needle type NO and RSNO sensors that possess the small size required for measuring NO/RSNO levels in SQ fluid of rats.

- Fabricated NO release SQ glucose sensors that exhibit much longer-term NO release (1 week) than previously thought to be possible, while maintaining good sensitivity and linearity toward glucose.

- Fabricated first needle-type glucose sensors with NO generating Cu(II)-cyclen-PU coatings and demonstrated that surfaces can generate NO in the presence of RSNO species, but that linearity of response toward glucose at the high end is not maintained.

-

REPORTABLE OUTCOMES

Conference presentations:

-J. A. Bennett, M. E. Meyerhoff, "Development of Advanced Electrochemical Sensors for NO and RSNO Detection in Subcutaneous Tissue," Midwestern Universities Analytical Chemistry Conference, November 2007, Champagne, IL.

-J. A. Bennett, M. E. Meyerhoff, "Electrochemical Needle-Type Sensors for Subcutaneous Detection of Nitric Oxide and *S*-Nitrosothiols," Pittsburgh Conference, March 2008, New Orleans, LA.

-Q. Yan and M. E. Meyerhoff, "Preparation and Characterization of Needle-Type Glucose/Lactate Sensors with Nitric Oxide Releasing/Generating Polymeric Coatings for Enhanced Biocompatibility," Poster, Pittcon Conference & Expo, March 3, 2008, New Orleans, LA.

-Q. Yan and M. E. Meyerhoff, "Needle-Type Glucose/Lactate Sensors with Nitric Oxide Releasing/Generating Polymeric Coatings for Enhanced Biocompatibility," Oral Presentation, The Tenth World Congress on Biosensors, May 16, 2008, Shanghai, China.

-J. Yang, J. L. Welby, M. E. Meyerhoff, "Generic Nitric Oxide (NO) Generating Surface by Immobilizing Organoselenium Species via Layer-by-Layer Assembly." The 8th World Biomaterials Congress, May 28, 2008, Amsterdam, The Netherlands.

-B. Wu, M.E. Meyerhoff, "Selenium-Derivatized Polyurethanes: Potential Nitric Oxide Generating Coatings for Stents and Other Biomedical Devices." The Society for Biomaterials 2008 Translational Biomaterial Research Symposium, Sept 11, 2008, Atlanta, GA.

Publications:

-Y. Wu and M. E. Meyerhoff, "Nitric Oxide Releasing/Generating Polymers for the Development of Implantable Chemical Sensors with Enhanced Biocompatibility, Talanta, 75, 642-650 (2008).

-S. Hwang and M. E. Meyerhoff, "Organoditelluride-Tethered Polymers that Spontaneously Generate Nitric Oxide when in Contact with Blood," J. Mater. Chem., 18, 1784-1791 (2008).

-S. Hwang and M. E. Meyerhoff, "Polyurethanes with Tethered Copper(II)-Cyclen Complex: Preparation, Characterization and Catalytic Generation of Nitric Oxide from S-Nitrosothiols," Biomaterials, 29, 2443-2452 (2008).

CONCLUSIONS

During the past year, new polyurethane materials were prepared that have immobilized Cu(II)-cyclen and RSe sites within their structure. These materials catalytically generate NO from RSNOs species present in solution at physiological pH as detected chemiluminescence. Further, we examined the potential for utilizing RTe species immobilized in a hydrogel polymer to serve as an NO generating coating material and found this material to be equally as catalytic as immobilized RSe type polymers. However, efforts to create improved coatings for SQ glucose sensors will remain focused on the new Cu(II) and RSe-based systems, including the use of a novel layer-by-layer self assembly approach to immobilize RSe and potentially Cu(II)-cyclen sites on the surface of implantable sensors. Indeed, while *in vivo* testing of these new NO materials was slowed during the past 12 months due to personnel and protocol changes, animal studies to examine the inflammatory behavior of these new materials when placed under the skin of rats has recently been restarted so we can reach a final conclusion on the effectiveness of NO generating polymers in reducing inflammatory response in the SQ space. We are also anxious to test whether there are adequate levels of RSNOs in the SQ tissue via use of our new needle type RSNO sensor developed over the past year. These studies will take place over the coming year. At the same time, we will continue to test the new NO generating coatings, as well as the new design for using NO release coatings on the surface of electrochemical needle type glucose sensors. Given the known effectiveness of the NO release strategy, our recent findings that we can prepare sensors that function well and also release NO for up to 7 days gives us a promising back-up approach to resolve SQ biocompatibility issues, should the levels of RSNO in the SQ space prove to be inadequate for ample NO generation.

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